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Autographa californica multiple nucleopolyhedrovirus GP64 protein: Analysis of domain I and V amino acid interactions and membrane fusion activity

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ABSTRACT

The *Autographa californica* multiple nucleopolyhedrovirus GP64 is a class III viral fusion protein. Although the post-fusion structure of GP64 has been solved, its pre-fusion structure and the detailed mechanism of conformational change are unknown. In GP64, domain V is predicted to interact with two domain I segments that flank fusion loop 2. To evaluate the significance of the amino acids involved in these interactions, we examined 24 amino acid positions that represent interacting and conserved residues within domains I and V. In several cases, substitution of a single amino acid involved in a predicted interaction disrupted membrane fusion activity, but no single amino acid pair appears to be absolutely required. We identified 4 critical residues in domain V (G438, W439, T452, and T456) that are important for membrane fusion, and two residues (G438 and W439) that appear to be important for formation or stability of the pre-fusion conformation of GP64.

Introduction

Membrane fusion is essential for entry of enveloped viruses into their host cells (Plemper, 2011). The process of membrane fusion is typically executed by one or more viral fusion proteins that are anchored in the envelope of the virus particle. Viral fusion proteins have been categorized into three distinct structural classes (Class I, II, and III) (Li and Modis, 2014; White et al., 2008). Even though viral fusion proteins within each class may have substantially different structures and show significant structural divergence, the proteins within each class appear to catalyze membrane fusion via a common mechanism (Martens and McMahon, 2008; White et al., 2008). Following triggering by receptor binding and/or low pH (or perhaps other mechanisms), the typically trimeric fusion proteins extend and expose a hydrophobic domain (a fusion peptide or fusion loops), which then associates with or inserts into the cellular membrane bilayer. The viral fusion protein next folds back onto itself and thereby draws the fusion peptide (and associated host membrane) into close proximity to the transmembrane domain of the fusion protein. The two separate membranes are thus brought into close proximity, facilitating the union of viral and cellular membranes. During the conformational changes that catalyze membrane fusion, the viral fusion protein forces the two membranes to progress through several stages of close contact and interaction. One experimentally distinct intermediate step is referred to as hemifusion (White et al., 2008). Hemifusion is a partial fusion that results from the merging of the outer leaflets of the two adjacent membrane bilayers, while the inner leaflet of each bilayer remains undisturbed. Hemifusion is followed by the rapid opening and closing of a small fusion pore which subsequently enlarges until a stable pore forms (White et al., 2008). Further expansion of the fusion pore completes the process, ultimately releasing the nucleocapsid core of the virus particle into the cell cytoplasm during viral entry.

Baculoviridae is a family of enveloped, double-stranded DNA viruses with circular genomes of approximately 80–180 kbp. Baculoviruses are infectious only to invertebrates, and viruses classified within this group have been isolated from species within the insect Orders Lepidoptera, Diptera, and Hymenoptera (Rohrmann, 2013). The most intensively studied baculovirus is *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Rohrmann, 2013). Budded virions (BVs) of AcMNPV enter host cells by clathrin-mediated endocytosis (Long et al., 2006). The major envelope glycoprotein of AcMNPV BVs, GP64, plays essential roles in virus attachment and fusion during entry, and is also important in virion egress by budding (Hefferon et al., 1999;

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Oomens and Blissard, 1999). GP64 is a classical type I membrane protein that is both necessary and sufficient for mediating pH-dependent membrane fusion during viral entry (Blissard and Wenz, 1992). The transmembrane domain and the pre-transmembrane domain (PTM)/stem region of this protein are critical for membrane fusion and virus infectivity (Li and Blissard, 2008, 2009a, b). GP64 has receptor binding activity (Hefferon et al., 1999), and the receptor binding domain was mapped to the N-terminal 160 aa (Zhou and Blissard, 2008b). Recently, a cholesterol recognition amino acid consensus domain and a pH-sensitive heparin-binding motif were both found to facilitate binding of GP64 to mammalian cells (Luz-Madrigal et al., 2013; Wu and Wang, 2012). Based on the crystal structure of the low-pH (post-fusion) form of AcMNPV GP64, GP64 proteins are classified as class III viral fusion proteins (Kadlec et al., 2008). Class III fusion proteins also include rhabdovirus envelope proteins (such as vesicular stomatitis virus G and rabies virus G), glycoprotein B or gB from herpesviruses, and the GP75 proteins from Thogotovirus-like orthomyxoviruses (Backovic and Jardetzky, 2011; Backovic et al., 2009; Heldwein et al., 2006; Kadlec et al., 2008; Roche et al., 2006; Roche et al., 2007). In addition, computational predictions also suggest that the Bornavirus envelope protein G may also be a class III fusion protein (Garry and Garry, 2009). Although class III viral fusion proteins belong to unrelated viral families and have no apparent sequence similarities, they share common structural features that are essential for membrane fusion, including a long-central helix that forms a triple-stranded coiled-coil at the heart of the trimer, and internal fusion loops (Backovic and Jardetzky, 2011). The hydrophobic residues within the fusion loops (loop 1 and 2) of GP64 are critical for membrane interaction and the progression of different stages of membrane fusion (Dong and Blissard, 2012; Li and Blissard, 2011).

In their post-fusion structures, class III fusion proteins are composed of five structural domains (Domains I–V) (Backovic and Jardetzky, 2011). Unlike the post-fusion structures of class I fusion proteins , in which a 6-helix bundle is proximal to the host and viral membrane-interacting end of the trimer, the post-fusion structure of GP64 has no 6-helix bundle and the long central triple-stranded coiled coil is separated from the fusion loops and transmembrane domain by the entirety of domain I and a portion of domain V (Backovic and Jardetzky, 2011; White et al., 2008) (Fig. 1A and B). GP64 domain V (Fig. 1A, DV) contains an alpha helix (helix D), and a downstream linker region that connects helix D to the pre-transmembrane (PTM) domain (Backovic and Jardetzky, 2011). Helix D and the adjacent downstream linker region of domain V interact extensively with two regions adjacent to fusion loop 2 in domain 1 (referred to as fusion loop 2-proximal segments 1 and 2, or FL2PS1 and FL2PS2) (Kadlec et al., 2008) (Fig. 1B a and b). We hypothesize that these interactions between domains I and V may collaborate to create and/or to stabilize the post-fusion structure, following the pH-triggered rearrangement of the protein trimer. To examine this hypothesis, we substituted residues that make contacts between domain V and domain I and investigated the effects of those substitutions on GP64-mediated membrane fusion.

Results

Construction and expression of modified GP64 proteins

To determine which GP64 residues within the C-terminal domain V portion of GP64 (Fig. 1A; DV in red) are important for membrane fusion activity, we first performed an analysis of the GP64 post-fusion (low pH) structure (Kadlec et al., 2008) to predict amino acid side-chain contacts between residues within the C-terminus of domain V and residues from other domains. Contacts were examined using the WHAT IF molecular modeling package (http://swift.cmbi.ru.nl/whatif/). In the crystal structure of the post-fusion GP64 trimer, helix D and the linker region (from domain V) are predicted to interact extensively with two segments (amino acids residues 144–149 and 163–189) that flank fusion loop 2 of Domain I (Fig.1A, C). Most of these interactions are predicted to occur within the same protomer (monomer) of GP64. These include interactions between the following residue pairs: L189-W439, K170-S437, R163-Q445, and L144-T452 (Fig. 1B). Other predicted interactions include: K146-M453, Q148-K457 and N149-G459 which are between the linker region downstream of helix D (in domain V) and residues flanking the FL2 loop region (Fig. 1B b and d). In contrast to those predicted interactions within each protomer, we also identified additional predicted interactions (K446-S447 and K146-E454), which are found between two separate protomers (Fig. 1B c and d). We performed a sequence alignment of these interacting regions of GP64 proteins from different baculoviruses, as well as the GP64-family homolog from *Thogotovirus*, GP75 (Morse et al., 1992) (Fig. 1C). Based on structural analysis of AcMNPV GP64 (above) and sequence conservation among these GP64 family proteins, we experimentally examined a number of selected amino acid positions. The selected positions include the predicted interacting residues described above (Fig. 1C, closed circles), and several of the highly conserved residues within helix D and the downstream linker region (Fig. 1C, open circles). All of the residues selected for analysis were either individually substituted with alanine, or pairs of interacting residues were substituted with alanines. Alanine was selected for substitutions as alanine is a small non-polar amino acid that contributes only minimally to hydrophobicity and thus represents a neutral substitution. Since substitutions within amino acid positions 432-440 may abolish the AcV5 epitope of GP64 (Hohmann and Faulkner, 1983; Monsma and Blissard, 1995), a c-Myc epitope tag was inserted between the signal peptide and the mature ectodomain of GP64 (Zhou and Blissard, 2008a) in constructs Myc-S437A, Myc-G438A, Myc-W439A, Myc-S440A, Myc-K170A/S437A, and Myc-L189A/W439A. It was previously demonstrated that GP64 containing a c-Myc tag at the N- terminus of the mature ectodomain is expressed and efficiently transported to the cell surface (Zhou and Blissard, 2008a), and we show here that fusion activity by the epitope tagged WT GP64 is similar to that of WT GP64 (see below).

Expression of wild-type and modified GP64 proteins in Sf9 cells was carried out by transient transfection and was driven by the promoter of the AcMNPV *ie1* gene. At 36 h post transfection, GP64 proteins from cell lysates were examined by Western blot analysis under either reducing or non-reducing conditions for SDS-PAGE (Fig. 2A). Non-reducing conditions were used to detect the GP64 trimer, in which monomers are covalently linked by disulfide bonds (Li and Blissard, 2010; Oomens et al., 1995). The same oligomeric forms (trimer I, trimer II and dimer) that are typically observed in infected cells, were detected from all GP64 proteins containing substitution mutations, except construct Myc-K170A/S437A (Fig. 2A). The intensities of bands corresponding to the WT and modified (amino acid substituted) GP64s were similar, indicating that the single or double substitution mutations did not substantially alter the expression, stability, or oligomerization of these GP64 proteins (Fig. 2A). The single exception to this observation was the double substitution construct Myc-K170A/S437A, which was not detected on either reducing or non-reducing gels (Fig. 2A), implying that the interaction between these two residues may be critical for the folding or stability of GP64.

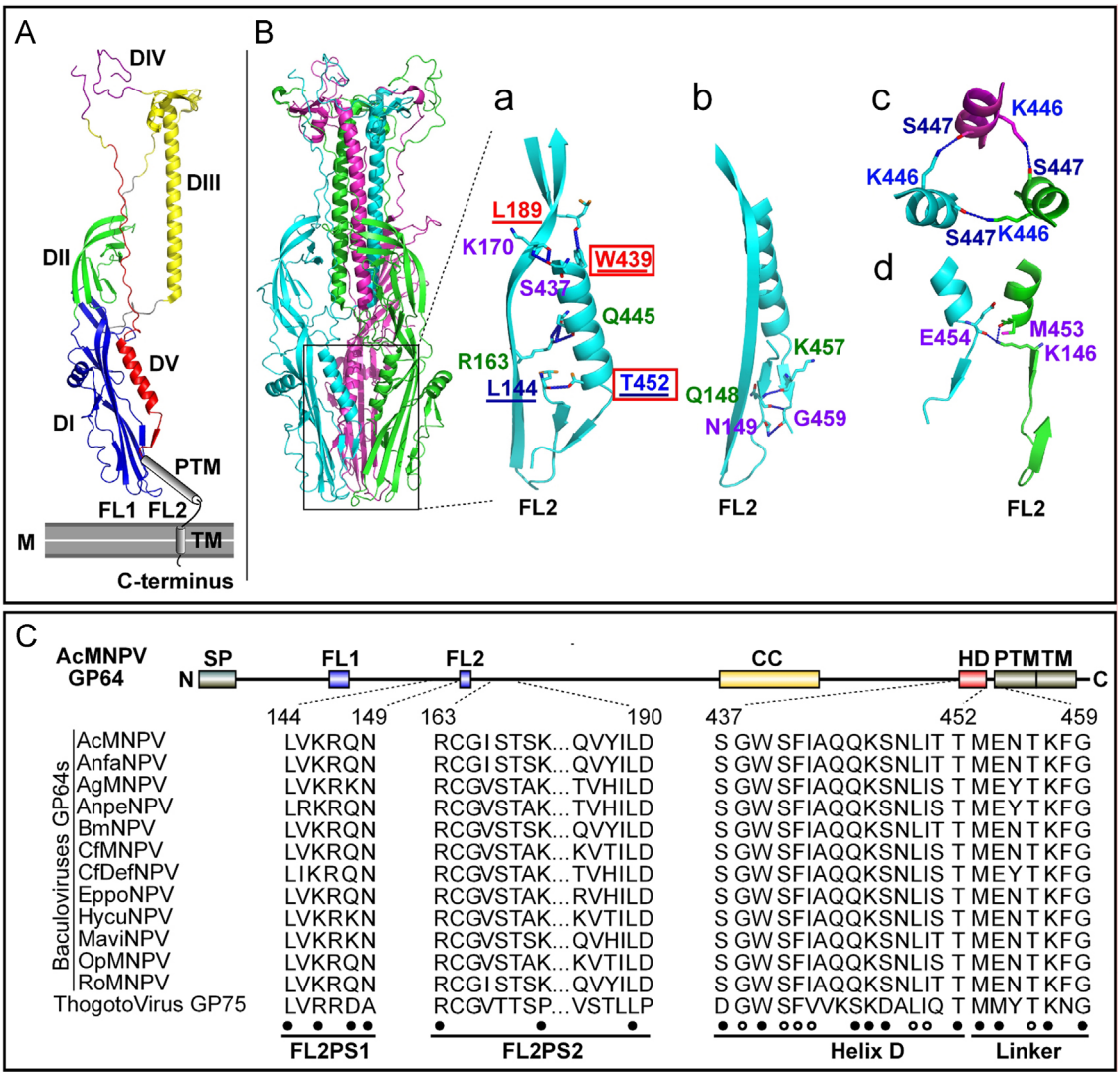

Fig. 1. Amino acid contacts and conservation in fusion loop 2-proximal segments and the C-terminus of domain V of AcMNPV GP64. (A) Post-fusion structure of GP64 monomer (PDB ID: 3DUZ). Domains I–V (DI–DV) are colored as blue, green, yellow, magenta, and red, respectively. The pre-transmembrane domain (PTM) and transmembrane domain (TM), which are absent in the post-fusion structure of GP64, are illustrated in gray in association with and proximal to the membrane (M). FL1 and FL2 indicate the positions of fusion loop 1 and fusion loop 2, respectively. (B) The GP64 post-fusion trimer is illustrated along with several expanded views (a–d) showing specific contacts between specific amino acid side-chains from fusion loop2-proximal segments and the C-terminus of domain V (a, b, d), or between two protomers (c, d). (C) Amino acid sequence alignments of fusion loop 2-proximal segments (FL2PS1 and FL2PS2) and the C-terminus of domain V, from baculovirus GP64 proteins and the Thogotovirus GP75 protein. The positions selected for amino acid substitution mutations are indicated as circles or large dots below the alignments (black dots represent interacting residues; open circles represent conserved residues). A linear schematic of the GP64 sequence is shown at the top of the alignments. SP, signal peptide; FL1, fusion loop1; FL2, fusion loop 2; CC, coiled coil; HD, helix D. Amino acid pairs representing contacts in the post-fusion structure are indicated in the same color. Amino acid pairs in which substitution of one residue resulted in loss of fusion activity are underlined, and the residue identified as critical for fusion activity is boxed in red. Virus abbreviations are as follows: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), Anagrapha falcifera NPV (AnfaNPV), Anticarsia gemmatalis MNPV (AgMNPV), Antheraea pernyi NPV (AnpeNPV), Bombyx mori NPV (BmNPV), Choristoneura fumiferana MNPV (CfMNPV), Choristoneura fumiferana DEF NPV (CfDefNPV), Epiphyas postvittana NPV (EppoNPV), Hyphantria cunea NPV (HycuNPV), Maruca vitrata MNPV (MaviMNPV), Orgyia pseudotsugata MNPV (OpMNPV), Rachiplusia ou MNPV (RoMNPV).

Cell surface localization of GP64 proteins with substitution mutations

Because the analysis of GP64 fusion activity requires GP64 localization to the plasma membrane, we examined the cell surface levels of the modified GP64 proteins using a cELISA assay, as described earlier (Li and Blissard, 2008). The relative cell surface level of each protein was measured and compared to that detected from WT GP64 (Fig. 2B, C, D, supplemental Table S2). Of the 12 amino acid positions examined in helix D of domain V, substitutions in 8 positions resulted in near WT levels of cell surface GP64. However, severely reduced cell surface levels were observed for constructs with substitutions in 4 positions of helix D (Fig. 2B; F441A, I442A, K446A, T452A). Of the 5 substitutions in the downstream linker region of domain V, 2 substitution constructs were present at the cell surface at near WT levels, and 3 substitutions (M453A, T456A, and G459A) resulted in dramatically reduced cell surface levels (Fig. 2B, Table S2). Similarly, low cell surface levels were also detected for 2 of the 6 double substitution constructs (Fig. 2C and D; L144A/T452A and N149A/G459A).

For GP64 constructs that were detected at the cell surface, but at levels below 10% of that from WT GP64, we also used indirect immunofluorescence to confirm GP64 at the cell surface. For these studies, GP64-expressing cells were fixed and GP64 was detected with monoclonal antibody AcV1, an antibody that recognizes the native neutral-pH conformation of GP64 (Hohmann and Faulkner, 1983; Zhou and Blissard, 2006). The results indicated that for all of those constructs expressing GP64 at less than 10% of the WT GP64 surface levels, the GP64 proteins were present at the surface and

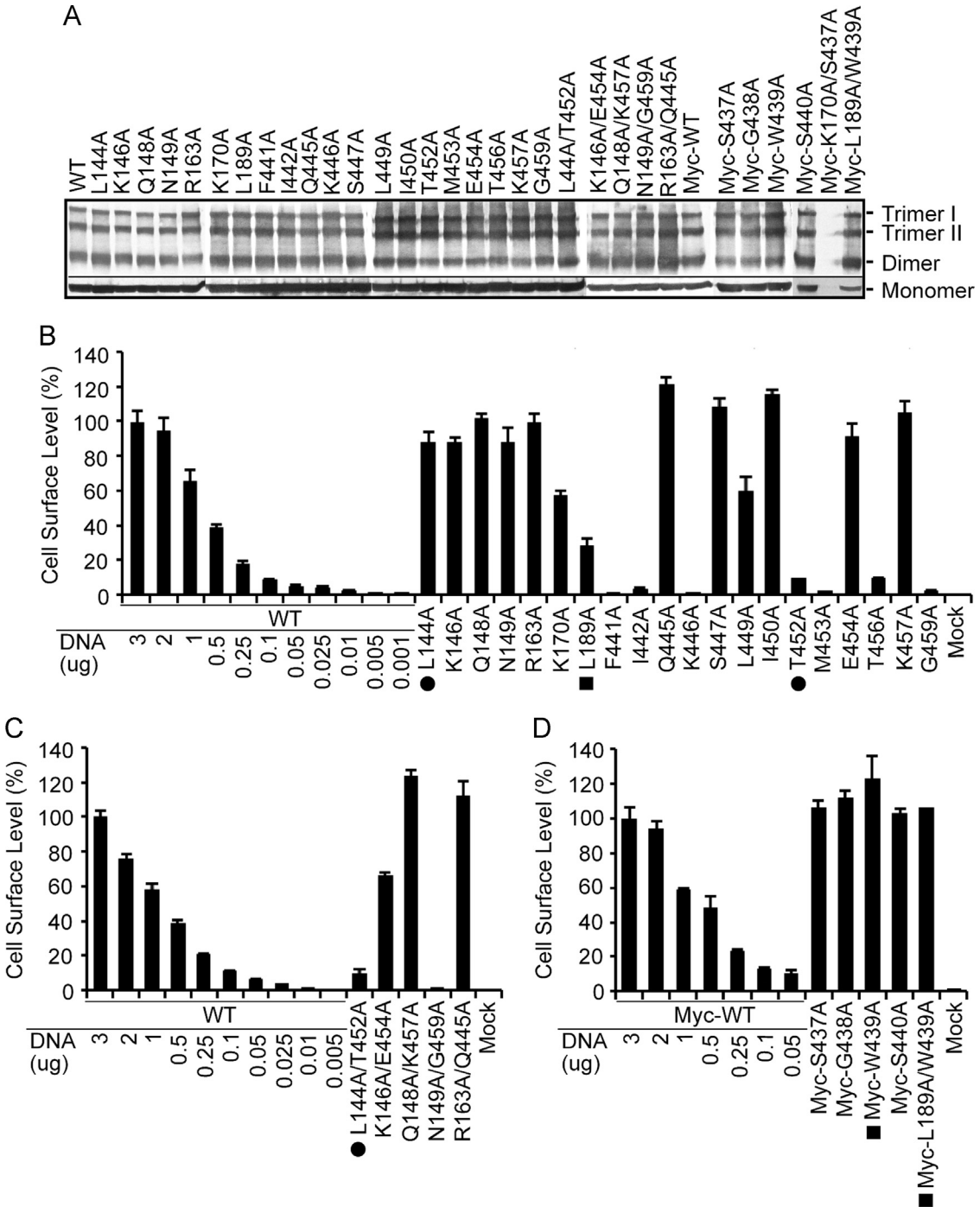

Fig. 2. Analysis of expression and cell surface localization of wild-type and modified GP64 proteins. (A) Western blot analysis of expression of wild-type and modified GP64 proteins under non-reducing (upper panel) and reducing (bottom panel) conditions. Sf9 cells were transfected with 3 μg of plasmid DNA, for expression of each modified GP64 construct, indicated above each lane. At 36 hp.t., GP64 protein expression was detected using anti-GP64 (MAb AcV5) or anti-Myc antibodies. The oligomerization state of GP64 protein is indicated on the right side of the panels. (B, C, D) Relative cell surface levels of wild-type and modified GP64 proteins were measured by cELISA using MAb AcV5 (B, C) or anti-Myc (D). A parallel standard curve generated by transfecting Sf9 cells with increasing quantities of the wild-type GP64-expressing plasmid is shown on the left side of each panel, while the cell surface level for each modified GP64 construct is shown on the right side of each panel. Cells were transfected with 3 μg plasmid DNA encoding and expressing each modified GP64 construct. Error bars represent the standard deviations from the mean of triplicate transfections for each construct. Constructs of amino acid substitutions in which one member of the pair resulted in loss of membrane fusion activity are indicated by dots (L144A/T452A) or squares (Myc-L189A/W439A).

AcV1 binding indicated that they were in the native pre-fusion conformation (data not shown).

Fusion activity of modified GP64 proteins

To determine whether substitution mutations that disrupt the interactions between residues flanking fusion loop 2 and residues in domain V, affect the fusion activity of GP64, we measured the fusion activities of wild-type and modified GP64 proteins in a cell-cell fusion assay. Because cell surface levels varied between different transiently-expressed GP64 constructs, we initially established a standard curve for cell surface levels of WT GP64 by transfecting Sf9 cells with decreasing quantities of the plasmid expressing wild-type GP64, and performing cELISA analysis

(Fig. 2B, C, D, left side of each panel). The relative fusion activity of each modified GP64 construct was compared with that from WT GP64 that was localized to the cell surface at an equivalent level, and a normalized fusion efficiency was calculated. A variety of the alanine substitution constructs mediated membrane fusion with an efficiency similar to that of WT GP64 (Fig. 3, Table S2). Most importantly, some GP64 constructs containing substitutions that disrupted the domain I-domain V interactions resulted in loss of fusion activity. Those constructs that disrupted fusion activity can be subdivided into two groups: The first group (Myc-G438A, Myc-W439A, and Myc-L189A/W439A) consists of constructs that were localized to the cell surface at near WT levels, but had no detectable fusion activity (Figs. 2D and 3B, Table S2; Myc-G438A, Myc-W439A, and Myc-L189A/W439A). Thus, the effects of these substitutions on fusion activity of the GP64 protein were severe. The second group contains constructs that were localized to the cell surface less efficiently than WT, and for which the normalized fusion efficiency was dramatically reduced but not absent (Figs. 2B, C and 3 B, Table S2; T452A, T456A, and L144A/T452A). For constructs in this second group, normalized fusion activities ranged from 2 to 21% as compared with WT GP64 (Fig. 3B; T452A, T456A, and L144A/T452A).

Thus, while a number of contacts between the fusion loop 2-proximal sequences and residues in and near helix D were not necessary for membrane fusion activity of GP64, we identified 2 residues in this region (G438 and W439) that were essential for fusion activity. In addition, we found that 2 additional residues (T452 and T456) appear to play an important role in fusion (Fig. 3B). Of the four important residues identified here, two (W439 and T452) are clearly involved in predicted interactions between domain V and domain I in the post-fusion structure (Fig. 1B a). Indeed, the double substitution mutation that includes T452 as well as its interacting residue (L144, immediately upstream of fusion loop 2) also resulted in a severe effect on membrane fusion (Fig. 1B a and Fig. 3B; L144A/T452A). It is of particular interest that these residues are found just upstream of the PTM and TM of the GP64 protein, and at opposite ends of helix D (Fig. 1A and B; DV). The observation that substitution of W439 and T452 resulted in complete absence of detectable fusion, and these residues are components of predicted interactions, suggest that the interactions may be necessary for catalyzing or stabilizing the close proximity of the two membranes which are associated with fusion loop 2 (FL2 in domain I) and the transmembrane domain, respectively. It is not clear why substitution of the interacting residues L144 and L189 did not also affect fusion activity, as might be expected. However, it is possible that either alternative interactions may form, or that each single interaction is not critical for membrane fusion. The other important residues (G438 and T456) identified in this analysis of membrane fusion, are highly conserved residues within the baculovirus and thogotovirus-like GP64 family proteins. However, their precise roles in the function of GP64 are less clear although we might speculate that they may be responsible for providing flexibility or stabilizing the pre- or post-fusion structure.

Analysis of membrane merger and pore formation

After identifying a number of amino acid positions that were important for membrane fusion, we next examined the step that was restricted in the fusion process for those GP64 constructs with inhibited fusion. Using a membrane-specific dye and a cytosolic dye, it is possible to experimentally dissect two of the steps in membrane fusion: outer membrane leaflet merger and pore formation (Li and Blissard, 2008; Ujike et al., 2004). For those GP64 constructs that were surface localized and did not induce fusion, or for which fusion efficiency was substantially reduced, we employed a previously described dye-transfer assay (Li and Blissard, 2008) to determine which step in membrane fusion was affected. Red blood cells (RBCs) were co-labeled with a membrane lipid dye (R18) and a cytosolic dye (calcein-AM), then bound to Sf9 cells expressing each GP64 construct at the cell surface. After low-pH triggering by incubation at pH 5.0, the transfer of each dye between RBCs and transfected Sf9 cells was monitored and efficiencies were calculated. Based on the transfer of the membrane and cytosolic dyes, each GP64 construct with a fusion defect can be classified as either: a) inhibiting outer leaflet merger (hemifusion), b) inhibiting pore formation, or c) reducing the efficiency of pore formation or expansion. For these studies, we examined the constructs that were expressed and localized at the cell surface, and had highly reduced or absent fusion activity. The selected constructs include Myc-G438A, Myc-W439A, T452A, T456A, L144A/T452A, and Myc-L189A/W439A. No dye transfer (neither R18 nor Calcein AM) was observed for constructs Myc-G438A, Myc-W439A, and Myc-L189A/W439A (Fig. 4A, lower right panels; 4B, Myc-G438A, Myc-W439A, Myc-L189A/W439A) suggesting that alanine substitution of the critical residues at the N-terminus of helix D (G438 and W439) disrupted the ability of GP64 to catalyze the initial merger of the outer membrane leaflets.

Constructs T452A, T456A, and L144A/T452A also showed a dramatic effect on membrane fusion but did not completely abolish fusion activity (Fig. 3), and these constructs appear to disrupt membrane fusion at a different step. For these constructs (T452A, T456A, and L144A/T452A) we observed transfer of both membrane (R18) and cytosolic (calcein-AM) dyes (Fig. 4A, lower left panels; 4B, T452A, T456A, and L144A/T452A), and dye transfer efficiencies were relatively low, approximately 3.7% (T452A), 11.5% (T456A), and 2.4% (L144A/T452A) (Fig. 4B). Positive and negative controls included cells with low GP64 surface levels and mock transfected cells. Note that Sf9 cells transfected with 0.001 μg of the plasmid expressing wild-type GP64 (positive control) showed readily detectable transfer of the two dyes and no dye transfer was detected from mock transfected cells (Fig. 4, WT0.001 vs Mock). Thus, these results suggest that for constructs T452A, T456A, and L144A/T452A, complete fusion pores formed, but at a substantially reduced rate or efficiency. The observation that constructs T452A, T456A, and L144A/T452A were found at the cell surface at reduced levels also suggests that these positions may be important for protein folding or stability, or transport.

Low-pH-induced conformational change in GP64 constructs

We identified several GP64 constructs that either induced fusion pore formation at a lower efficiency, or could not induce outer leaflet merger at the initial membrane fusion step after low pH triggering. To determine whether the fusion defect for those constructs was caused by a measurable effect on the low-pH triggered conformational change in GP64, we used a conformation-specific monoclonal antibody (MAb AcV1) that binds only to the neutral-pH (or prefusion) form of GP64. We examined binding of AcV1 to those GP64 constructs at various pH values. It was previously established that MAb AcV1 recognizes only the prefusion conformation of GP64 (Li and Blissard, 2011; Zhou and Blissard, 2006), and that the AcV1 epitope is lost upon the low-pH triggered conformational change (Zhou and Blissard, 2006). As shown in Fig. 5 (closed circles), successively lowering the pH of the medium from 7.0 to 4.5 resulted in dramatic loss of AcV1 binding to wild-type GP64 or an epitope-tagged wild-type GP64 construct (Fig. 5B, D, E, F, Myc-WT and WT). For all of the GP64 constructs examined (T452A, T456A, and L144A/T452A, Myc-G438A, Myc-W439A, and Myc-L189A/W439A) AcV1 binding was dramatically reduced in comparison to that for WT GP64. This suggests that even though fusion is affected at two different stages

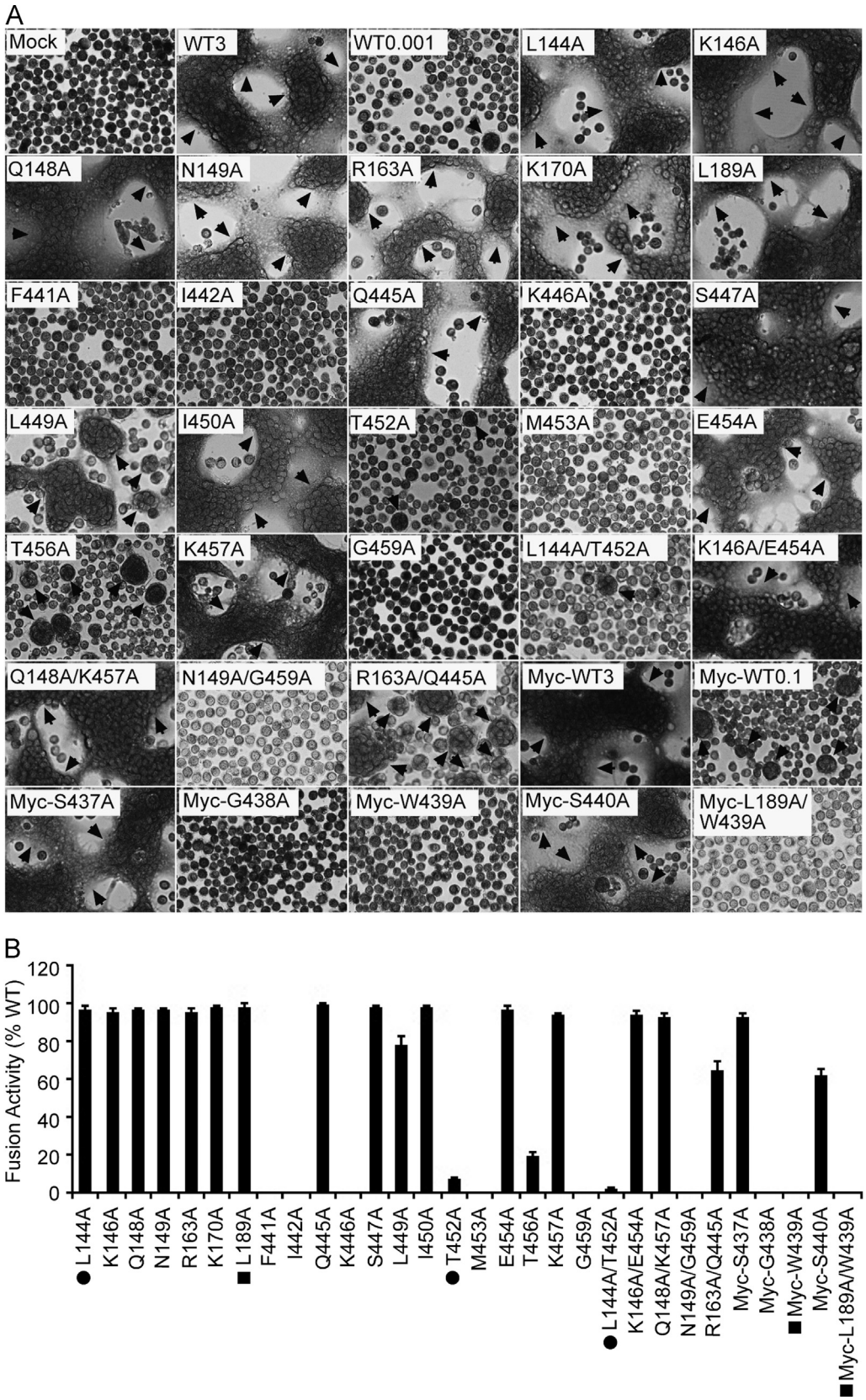

Fig. 3. Analysis of membrane fusion activity of wild-type and modified GP64 proteins. (A) Cell–cell syncytium formation assay. Sf9 cells were transfected with 3 μg (WT3) or 0.001 μg (WT0.001) of plasmid DNA from a WT-GP64-expressing plasmid. For analysis of modified GP64 proteins, cells were transfected with 3 μg plasmid DNA. Labels in each panel indicate the amino acid substitution(s). At 36 hp.t., the syncytium formation was observed and photographed under phase-contrast microscopy at 4 h after the low pH treatment. Arrows indicate syncytial masses. (B) Analysis of syncytium formation efficiency. The relative fusion activity for each construct was determined as described in the Materials and Methods section. Error bars represent the standard deviation from the mean of the results of triplicate transfections. Constructs of amino acid substitutions in which one member of the pair resulted in loss of membrane fusion activity are indicated by dots (L144A/T452A) or squares (Myc-L189A/W439A).

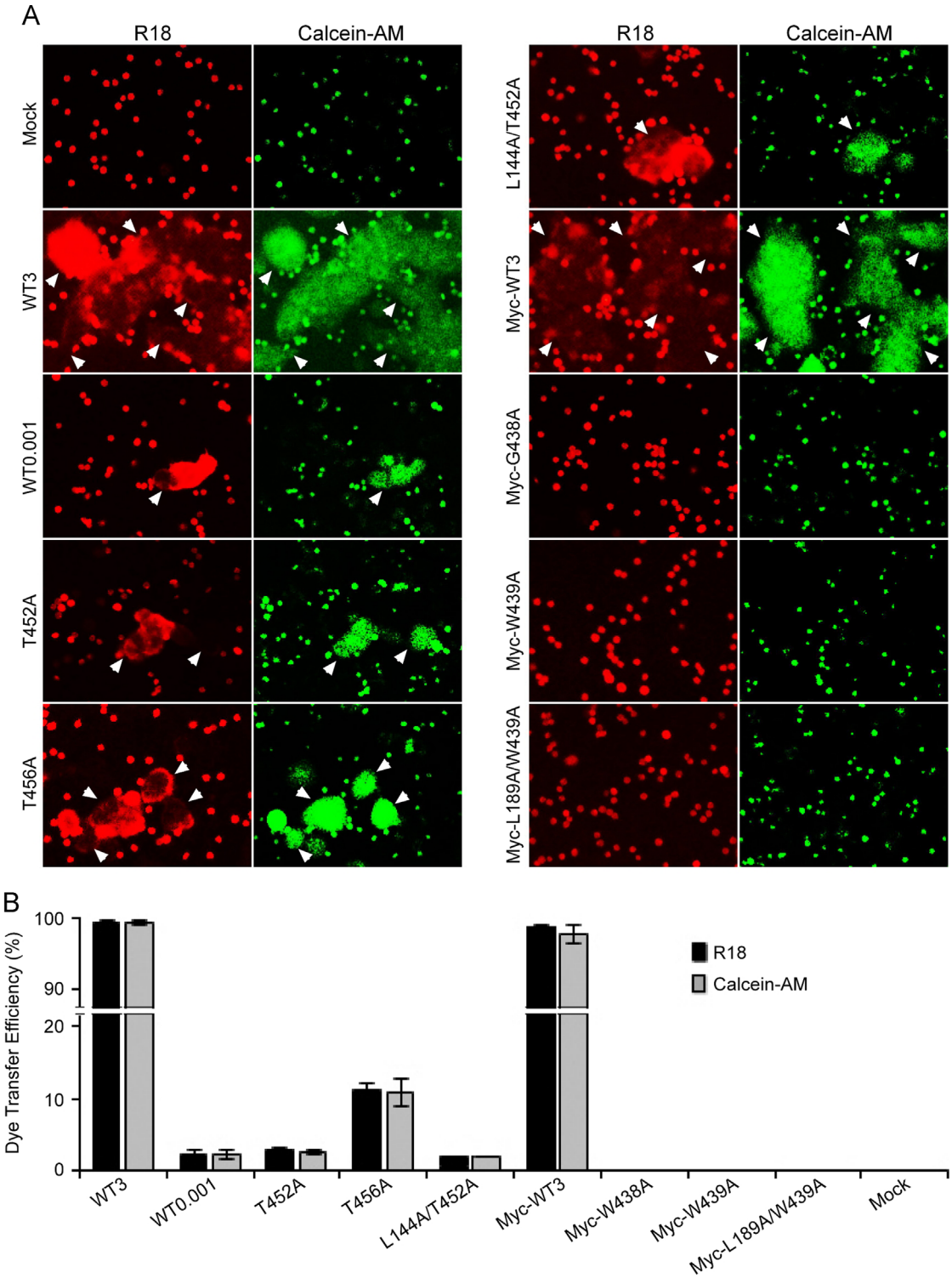

Fig. 4. Analysis of hemifusion and pore formation by fusion-deficient GP64 proteins. (A) Sf9 cells were transfected with 3 μg or 0.001 μgWT-GP64-expressing plasmid (WT3 or WT0.001) or with 3 μg plasmid DNA for each modified GP64 construct. At 36 hp.t., the transfected Sf9 cells were incubated with dual dye-labeling red blood cells (RBCs) and the dye transfer between the smaller RBCs (small cells) and the larger Sf9 cells were observed and photographed under fluorescence microscopy. The Sf9 cells containing the dye transferred from dual-dye labeled RBCs are indicated by arrows. (B) Analysis of dye transfer efficiency. The efficiency of lipid or cytosolic dye transfer was estimated from the ratio of the number of R18-transferred or calcein-AM-transferred Sf9 cells to the number of Sf9 cells with RBCs bound. Five fields were examined for each GP64 construct. Error bars represent the standard deviation from the mean of the results of triplicate transfections.

for different sets of constructs (Fig. 4), the prefusion conformation appears to be dramatically affected in both cases. It should be noted however that for constructs T452A, T456A, and L144A/T452A (Fig. 5D, E, F) a very modest pattern of loss of AcV1 binding was observed as pH values were lowered from 7.0 to 5.0. However, these results are difficult to interpret due to the lower levels of protein at the cell surface. In contrast, constructs Myc-G438A, Myc-W439A, and Myc-L189A/W439A (Fig. 5B) were present at the cell surface at levels similar to WT GP64, yet each showed dramatic loss of AcV1 binding at pH 7, clearly indicating loss of the

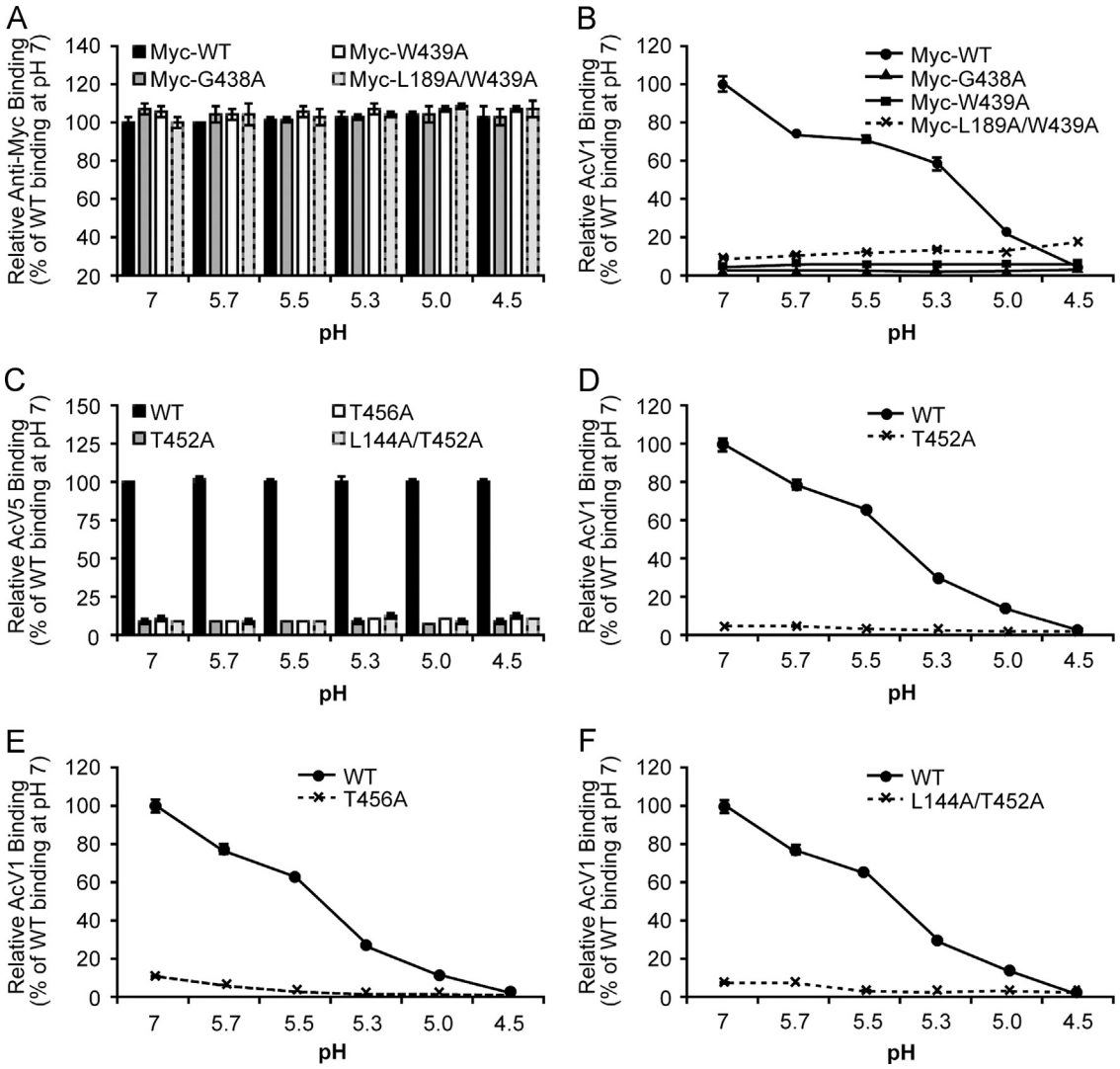

Fig. 5. Analysis of the pre-fusion conformation of WT and modified GP64 constructs. To examine the conformation of GP64 proteins at varying pH values, binding of anti-Myc (A), AcV5 (C) or the conformation-specific MAb of GP64, AcV1 (B, D, E, F), was measured as described in the Materials and Methods section. Sf9 cells were transfected with 3 μgWT-GP64-expressing plasmid (Myc-WT, WT) or with 3 μg plasmid for each modified GP64 construct. Each value represents the mean from triplicate transfections and is normalized to that of Sf9 cells transfected with wild-type GP64 expressing plasmid at neutral pH (pH 7).

pre-fusion structure in these modified protein constructs. Thus, some or all of these mutations may destabilize or prevent formation of the neutral-pH conformation of GP64.

Discussion

Biochemical and structural properties of class III viral fusion proteins (rhabdovirus G, herpesvirus gB, orthomyxovirus GP75, and baculovirus GP64 proteins), are distinct from both class I and class II viral fusion proteins (Backovic and Jardetzky, 2011). However, the available structural data suggest that class III viral fusion proteins may utilize a fusion mechanism that is similar to the trimer-of-hairpins mechanism that has been described as driving membrane fusion for class I viral fusion proteins (Backovic and Jardetzky, 2011; Kadlec et al., 2008; Kielian and Rey, 2006; Roche et al., 2007; White et al., 2008). In class I fusion proteins, two helical heptad repeat (HR) domains (HRA and HRB) are located adjacent to the fusion peptide and transmembrane domain, respectively. Upon fusion activation and conformational change, the central HRA coiled coil packs against the C-terminal HRB in an antiparallel manner. This conformational change in each of the three protomers of the trimer, forms the trimer of hairpins which is stabilized as a six-helix bundle (6HB)(White et al., 2008). In some class I fusion proteins, such as human immunodeficiency virus (HIV) GP41, the 6HB is long and extends close to the membrane (Weissenhorn et al., 1997). In other class I fusion proteins, such as influenza virus hemagglutinin (HA), the 6HB is relatively short and farther from the membrane (Bullough et al., 1994). In class II fusion proteins, the trimer of hairpins may be stabilized by interactions between adjacent beta sheets and unstructured regions, as opposed to the 6HB of class I fusion proteins (Kielian and Rey, 2006). The class III fusion protein, GP64, does not have a 6HB near the C- terminus of the post-fusion structure (like Class I proteins) but rather has one alpha helix near the C-terminus of each protomer (Fig. 1A, helix D), just upstream of the pre-transmembrane domain. Examination of the structure and interactions between that helix and sequences adjacent to fusion loop 2, suggested that those interactions may stabilize the hairpin that positions fusion loop 2 near the TM domain in the post-fusion structure of GP64. Helix D of domain V is located upstream of the transmembrane domain, and a number of specific residues within and flanking helix D (Fig. 1B a; T452, Q445, and S437) interact directly with residues that flank fusion loop 2 (Fig. 1B a; L144,

R163, K170, L189). Although these interactions were observed in the crystal structure, it was not yet clear whether the interactions between the C-terminus of domain V (helix D) and the fusion loop 2 region of domain I, were required for membrane fusion activity. To examine that question experimentally, we made amino acid substitutions in a variety of key positions that are predicted to mediate or contribute to the stability of the post-fusion structure, or are conserved in GP64 family proteins. Specifically, we examined 7 amino acid residues that flank fusion loop 2 (FL2), and 17 residues in or adjacent to helix D of domain V (Fig. 1C, open and closed circles). After excluding those constructs in which the substitutions resulted in artifacts (loss of expression or surface localization), we functionally examined each substituted GP64 construct. We identified amino acids are within the predicted interactions and that appear to be clearly important or required for GP64-mediated membrane fusion (Fig. 1B a, C and Fig. 3; W439 and T452). We also identified one conserved position (G438) that had a dramatic effect on membrane fusion.

A number of amino acids involved in contacts between the C-terminal region of domain V and fusion loop 2-proximal segments (Fig.1B a, b and d, and Fig. 1C, FL2PS1 and FL2PS2) did not appear to be important for membrane fusion. Single alanine substitutions of L144, K146, Q148, N149, and R163 (which are located within the loop region proximal to the N- and C-terminus of fusion loop 2), had no apparent effect on expression, cell surface localization, or fusion activity of those modified GP64 proteins. It was found previously that a double alanine substitution of N149 and N150 resulted in a significant decrease in fusion activity (Dong and Blissard, 2012). Combined with the current results, we may now conclude that that effect of that double substitution may be largely due to the substitution at residue N150. In contrast to the effect of substitutions L144A, K146A, Q148A, N149A, and R163A (which did not inhibit fusion), alanine substitutions of all but one (Q445) of the corresponding contact residues within the C-terminus of domain V (T452A, M453A, K457A, and G459A) significantly reduced the cell surface levels of GP64 suggesting that T452, M453, K457, and G459 may be required for structural stability of the pre-fusion conformation of GP64.

Of the amino acids identified as important for fusion, two (T452 and W439) include amino acids that are involved in predicted contacts between domain V and domain I (Fig. 1, L144-T452 and L189-W439). However in each of these cases, when the opposing amino was substituted with alanine (L144A or L189A), little or no effect on fusion was observed. This result suggests that either a) the interaction itself is not required but one of the amino acids is required for a different function apart from the interaction, or b) the alanine substitution at one of the amino acid positions could result in the formation of a new alternative interaction. It is also possible that the general structural stability of this region of the molecule may be determined by multiple interactions between domains I and V and that no single interaction is indispensable for protein function, a strategy of structural redundancy. Thus a clear understanding may require simultaneous disruption of multiple predicted interactions in the structure of the molecule. Helix D of domain V is immediately upstream of a linker region that connects helix D with the pre-transmembrane domain (PTM) (Fig. 1B). Previous results indicated that the PTM domains of GP64 and herpesvirus gB are essential for membrane fusion and it has been proposed that the PTM domain may somehow collaborate with fusion loops to coordinate merger of the target membrane and viral envelope (Li and Blissard, 2009b; Shelly et al., 2012). Thus, the interactions that position helix D and the downstream linker and PTM domain (Fig. 1A) are likely to be important for membrane fusion activity.

Presently, VSV G is the only class III fusion protein for which both pre-fusion and post-fusion crystal structures have been solved (Roche et al., 2006; Roche et al., 2007). Distinct from class I and II fusion proteins, in which significant refolding of individual domains results in the conformation change (White et al., 2008), the domains of VSV G mostly retain their structure (Roche et al., 2007). During the low-pH triggered conformation change of G, repositioning of domains occurs through the flexible linker regions that connect domains, particularly a so-called hinge region (Roche et al., 2008; Roche et al., 2007). Additionally, the reversible conformation change of G and GP64 was proposed to be initiated by pH-induced protonation and deprotonation of several key histidine residues (Kadlec et al., 2008; Roche et al., 2008). Previous studies revealed that a cluster of histidine residues including H245, H304, and H430 are involved in the low-pH triggered conformation change of GP64. Triple alanine substitutions of these histidine residues resulted in a GP64 protein with an altered pre-fusion conformation as determined by loss of recognition by the conformation-specific monoclonal antibody AcV1 (Li and Blissard, 2011). Here, we observed a similar phenotype for constructs with alanine substitutions of G438, W439, or Myc-L189-W439, which are located at the N-terminus of helix D (G438 and W439) (Figs. 1, 5, 6A). Fusion activity for constructs with substitutions at G438 and W439 was undetectable (Fig. 3), and further examination by analysis of membrane and cytosolic markers for fusion indicated that the defect in membrane fusion occurred at a step prior to membrane merger (Fig. 4). Combined, these results suggest that the contacts and interactions of G438 and W439 at the end of helix D, are not required for expression or accumulation of GP64 at the cell surface, but are likely required for the normal pre-fusion conformation of GP64, and without this structure, the initial step in membrane fusion does not occur. Combined with prior data, the current studies of the potentially stabilizing effect of amino acid positions helix D and fusion loop proximal sequences, are consistent with a model in which the closely neighboring regions located at the base of the central coiled coil in the post-fusion structure of GP64 may represent a potential hinge region (Fig. 6) that is critical for conformational change and/or the stability of the pre-fusion structure of GP64.

In summary, we examined the predicted interactions between domain V and fusion loop 2 of the AcMNPV GP64 protein. Through the direct analysis of 24 amino acid positions that represent interacting and conserved residues within domains I and V, we identified 4 critical amino acid positions in domain V (G438, W439, T452, and T456) that are important for membrane fusion activity of GP64. Further analysis indicated that at least two of these positions (G438 and W439) appear to be important for formation or stability of the pre-fusion conformation of GP64. Importantly, of the single amino acid pairs that we identified as potentially mediating stabilizing interactions between domains V and I, no single interaction appears to be essential for fusion activity. However, some single amino acid substitutions within those pairs had profound effects on membrane fusion activity. It is possible that for those pairs, alternative interactions may form when one member was substituted, permitting fusion activity. Alternatively it is also possible that multiple single interactions stabilize the overall interaction between these two domains and any single interaction is dispensable. By better understanding how the pre- and post-fusion trimers of GP64 are formed or stabilized, these results represent an important step toward elucidating the mechanisms by which class III viral fusion proteins undergo a series of conformational states, ultimately bringing two adjacent membranes together and resulting in membrane fusion.

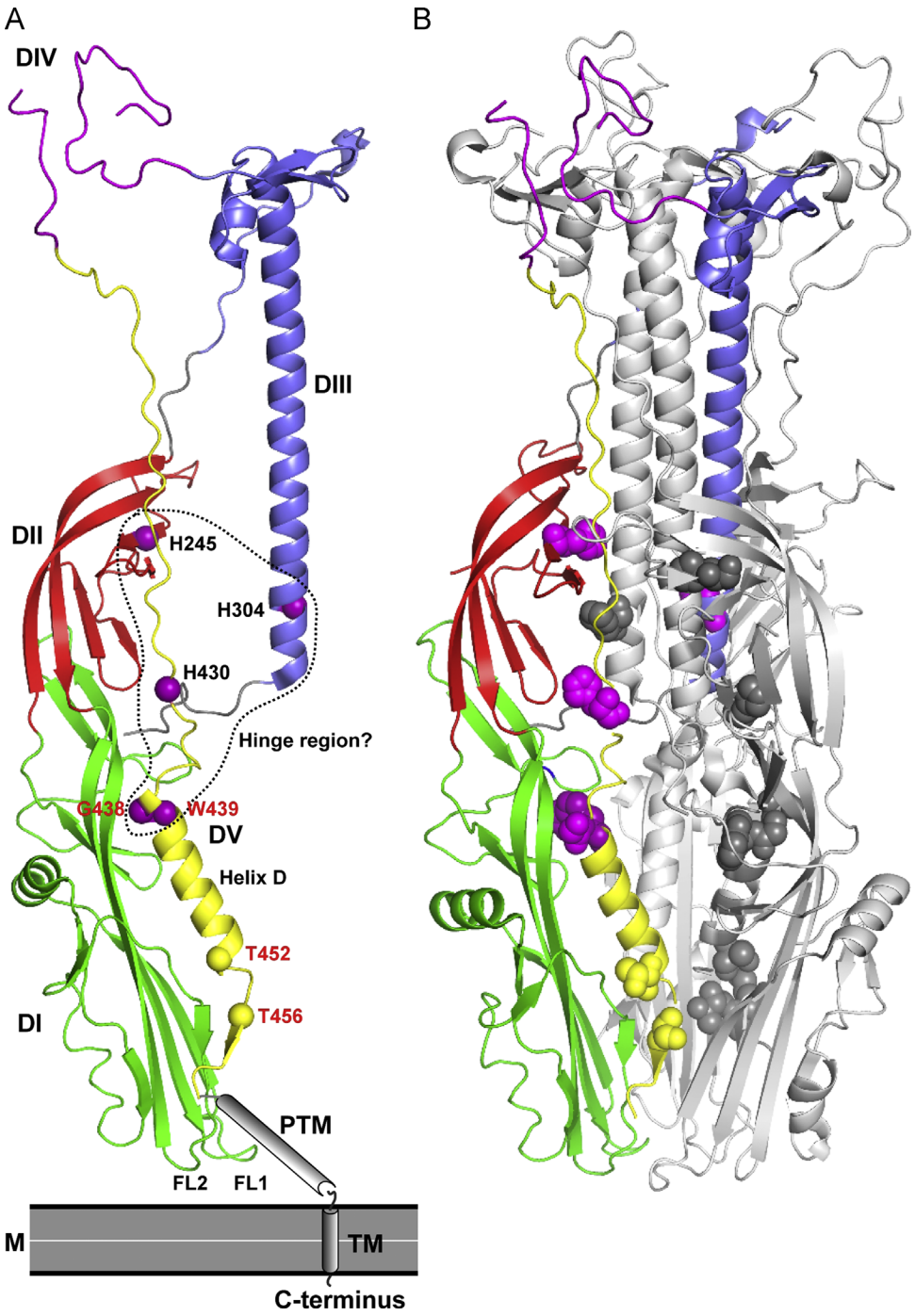

Fig. 6. Critical residues within the C-terminus of domain V of GP64 that are involved in GP64 conformation change and membrane fusion are indicated on monomeric (A) and trimeric (B) structures of GP64. (A) Domains I–V of the post-fusion structure of GP64 monomer are colored in green, red, blue, pink, and yellow, respectively. Residues identified in prior studies (Li and Blissard, 2011) and in this study, as essential for conformation change are indicated as magenta spheres. The residues essential for membrane fusion are labeled in red. The potential hinge region is encircled by a dashed line. (B) Domains I–V of the first protomer are colored as in (A), and the critical residues and their side chains are shown as space-filling structures colored as in (A). The same critical residues are shown as space-filling structures but are colored in gray in the second protomer. The second and third protomers are colored in gray.

Materials and methods

Cells and antibodies

Spodoptera frugiperda (Sf9) cells and a GP64-complementing cell line ($Sf9^{Op1D}$, a line that continuously expresses the Orgyia pseudotsugata (Op)MNPV GP64 protein)(Plonsky et al., 1999), were cultured at 27 °C in TNMFH medium containing 10% fetal bovine serum (FBS). Cells were transfected using CaPO4 precipitation as described previously (Blissard and Wenz, 1992). The primary monoclonal antibodies AcV1 and AcV5 against AcMNPV GP64 were purchased from Santa Cruz Biotechnology, and anti-Myc monoclonal antibody was from EarthOx, L.L.C.

Mutagenesis and construction of plasmids

The GP64 fragments encoding substitution mutations were generated by overlap PCR. The PCR primers are listed in Supplemental Table S1. The PCR products were digested with unique

restriction enzymes XbaI and NotI, or NotI and EcoRI, then subcloned into pBiepA (Li and Blissard, 2008). To generate Myc-tagged GP64, a c-Myc epitope tag (EQKLISEEDL) was inserted between the signal peptide and mature ectodomain of GP64 by overlap PCR as described previously (Zhou and Blissard, 2008a). Plasmid DNAs for transfections were isolated using a DNA Midiprep kit (Invitrogen). Constructs were confirmed by restriction enzyme digestion and DNA sequencing. The recombinant *gp64* knockout bacmid (*gp64*$^-$-GUS) was constructed by inserting a cassette containing a β-glucuronidase (GUS) gene under the control of the AcMNPV *p6.9* late promoter into the polyhedron locus of an AcMNPV *gp64*-null bacmid (Lung et al., 2002) by Tn*7*-mediated transposition (Luckow et al., 1993) as described previously (Li and Blissard, 2008). The *gp64*$^-$*-GUS* virus was grown and its titer was determined in Sf9^{OP1D} cells.

cELISA assay

The cell-surface-localized GP64 proteins were analyzed using a cell surface enzyme-linked immunosorbent assay (cELISA) as described previously (Li and Blissard, 2008). Briefly, Sf9 cells in 12-well plates (2×10^5 cells/well) were transfected with plasmid DNAs. At 36 h post transfection (p.t.), the transfected cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS, pH7.4) for 10 min. Relative levels of cell surface-localized GP64 were measured by using monoclonal antibody (MAb) AcV5 (anti-GP64) or anti-Myc combined with a secondary goat anti-mouse antibody conjugated to beta-Galactosidase. To determine whether modified GP64 proteins are capable of undergoing low-pH-induced conformational change, cELISA was performed with MAb AcV1, which recognizes the neutral-pH conformation of GP64 but not the low-pH conformation (Zhou and Blissard, 2006). For cELISA analysis with anti-Myc and AcV1, transfected Sf9 cells (at 36 hp.t.) were incubated for 30 min in PBS, adjusted to various pH values (4.5–7), and then fixed in 4% paraformaldehyde for 30 min. The subsequent steps in cELISA were similar to those used for MAb AcV5 as described previously (Li and Blissard, 2008).

Immunofluorescence

To confirm cell-surface localization of GP64, Sf9 cells in 12-well plates (2×10^5 cells/well) were transfected with plasmids expressing either wild-type (WT) or modified GP64 proteins. At 36 hp.t., cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min. Cells were washed once with PBS and then incubated with 1% gelatin blocking buffer (in PBS, pH 7.4) for 2 h at 27 °C. After washing once with PBS, the cells were incubated with AcV1 at 27 °C for 1 h. Cells were washed three times with PBS (pH 7.4) and incubated for 1 h at 27 °C with Alexa fluor 594-goat-anti-mouse (Molecular Probes, Invitrogen) diluted 1:750 in PBS. After washing five times with PBS (pH 7.4), florescence was observed with a Nikon Eclipse Ti epifluorescence microscope.

Syncytium formation and fusion assay

To compare the relative membrane fusion activity for different GP64 constructs, Sf9 cells in 12-well plates were transfected with plasmids encoding WT or modified forms of GP64. At 36 hp.t., the transfected cells were washed once with PBS (pH 7.4) and incubated in PBS at pH 5.0 for 3 min. Then the cells were washed with PBS at pH 7.4, and returned to TNMFH medium. After 4 h incubation at 27 °C, cells were fixed with methanol for 10 min. The cytoplasm and nuclei of fixed Sf9 cells were separately stained with 0.1% Eosin Y and 0.1% methylene blue, respectively, and the number of nuclei found in syncytial masses was scored. A syncytial mass was defined as a mass of fused cells containing at least five nuclei. Five randomly selected representative fields were evaluated for each construct. The relative levels of fusion activity were calculated as described previously (Li and Blissard, 2008). Briefly, the number of nuclei in syncytial masses was divided by the total number of nuclei in a field. Those percentages were then normalized to parallel syncytium formation data from wild type GP64 that was localized to the cell surface at equivalent levels.

Hemifusion and pore formation assay

To analyze hemifusion and pore formation by modified GP64 proteins, a dual dye transfer assay was performed as described previously (Li and Blissard, 2008). Briefly, sheep red blood cells (RBCs; Jiulong Biotechnology) were co-labeled with the membrane-restricted lipid probe (octadecyl rhodamine B chloride or R18) and the cytosolic aqueous dye calcein-AM (Molecular Probes, Invitrogen). At 36 hp.t., transfected Sf9 cells were washed twice with PBS (pH 7.4) and then incubated with the dual labeled RBCs for 20 min at room temperature (RT) to facilitate binding. Unbound RBCs were removed by washing three times with PBS (pH 7.4). Sf9 cells with bound RBCs were then incubated with PBS at pH 5.0 for 3 min at RT, washed in PBS at pH 7.4, and then transferred into TNMFH medium. After incubation for 20 min at 27 °C, the transfer of fluorescence between RBCs and Sf9 cells was observed using epifluorescence microscopy. Dye transfer efficiency was determined as described previously (Li and Blissard, 2011). Briefly, five randomly selected fields were scored for dye transfer. The efficiency of hemifusion was calculated as the ratio of R18 dye-transferred Sf9 cells to RBC-bound Sf9 cells. Similarly, the efficiency of pore formation was estimated as the ratio of calcein-AM dye-transferred Sf9 cells to RBC-bound Sf9 cells.

Western blot analysis

Transfected Sf9 cells were lysed with TritonX-100 buffer (150 mM sodium chloride, 0.1% Triton X-100, 50 mM Tris, pH 8.0) containing a protease inhibitor cocktail (Roche) and the extracted proteins were separated by reducing and non-reducing SDS-PAGE in 6% or 10% polyacrylamide gels, transferred to PVDF membrane (Millipore), and blots were blocked in a 4% milk TBST solution as described previously (Oomens and Blissard, 1999). GP64 proteins were detected by MAb AcV5 or anti-Myc at a dilution of 1:1000 and immunoreactive proteins were visualized using an alkaline phosphatase-conjugated goat anti-mouse IgG antibody and NBT/BCIP (Promega).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.025.

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